Plasmid 67 (2012) 155–161

Contents lists available at SciVerse ScienceDirect

Plasmid

journal homepage: www.elsevier.com/locate/yplas

In situ monitoring of IncF plasmid transfer on semi-solid agar surfaces reveals a limited invasion of plasmids in recipient colonies

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ARTICLE INFO

Article history:
Received 9 December 2011
Accepted 3 January 2012
Available online 10 January 2012
Communicated by Eva Top

Keywords:
Bacterial conjugation
Transitory derepression
IncF plasmid
Swarming
In situ monitoring
Reporter gene

ABSTRACT

Most natural conjugative IncF plasmids encode a fertility inhibition system that represses transfer gene expression in the majority of plasmid-carrying cells. The successful spread of these plasmids in clinically relevant bacteria has been suggested to be supported by a transitory derepression of transfer gene expression in newly formed transconjugants. In this study, we aimed to monitor the extent of transitory derepression during agar surface matings in situ by comparing plasmid spread of the IncF plasmid R1 and its derepressed mutant R1*drd*19 at low initial cell densities. A zygotic induction strategy was used to visualize the spatial distribution of fluorescent transconjugants within the heterogeneous environment. Epifluorescence and confocal microscopy revealed different transfer patterns for both plasmids, however, spread beyond the first five recipient cell layers adjacent to the donor cells was not observed. Similar results were observed for other prototypical conjugative plasmids. These results cannot rule out that transitory derepression contributes to the limited R1 plasmid invasion, but other factors like nutrient availability or spatial structure seem to limit plasmid spread.

1. Introduction

Many of the genes encoding extended-spectrum-beta-lactamases, quinolone or carbapenem resistance found in infecting *Enterobacteriaceae* that resisted antibiotic treatment are located on large plasmids (Queenan and Bush, 2007; Walther-Rasmussen and Hoiby, 2002). These plasmids with narrow host range often belong to the incompatibility groups IncF, IncH, and IncI (Canton et al., 2008; Coque et al., 2008; Hawkey, 2008; Novais et al., 2007) and encode type IV secretion systems that enable conjugative transfer to other enterics via mechanisms requiring sex-pilus elaboration and cell–cell contact (Llosa et al., 2009). Thus, horizontal gene transfer via bacterial conjugation is a major force driving the recent antibiotic resistance spread among *Enterobacteriaceae* (Su et al., 2008).

From an evolutionary point of view, the survival of plasmids as genetic units requires a positive balance of factors that favor dissemination, such as carriage of beneficial traits or the horizontal transmission via conjugation, and factors that lead to loss of plasmids in a population such as uneven segregation, the fitness costs of maintaining conjugative plasmids and the attack of sex-pilus specific bacteriophages (Bergstrom et al., 2000). As an effective adaptation to the disadvantages of constitutive conjugative gene expression, many natural conjugative plasmids have evolved complex control circuits that repress the expression of conjugation genes in the majority of plasmid carrying cells (Frost and Koraimann, 2010). The majority of native IncF and IncI plasmids possess such a fertility inhibition (Fin) system exemplified by the well studied IncFII paradigm plasmid R1 (Koraimann et al., 1991, 1996; Polzleitner et al., 1997). Laboratory derepressed

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0147-619X
doi:10.1016/j.plasmid.2012.01.001

(*drd*) mutant derivatives with dysfunctional Fin components support high conjugation frequencies by their hosts. However, other families of conjugative plasmids such as IncP and IncW plasmids do not repress conjugative gene expression and are considered to be naturally derepressed plasmids (Bradley et al., 1980).

Despite repression of conjugative gene expression, kinetic studies of plasmid transfer using laboratory strains in vitro revealed that even if repressed plasmids are introduced at low frequencies in a bacterial population, transconjugant cells that have acquired the plasmids rapidly dominate the population (Lundquist and Levin, 1986). Mathematical models fitted the experimental data best when the plasmid transfer rate of newly formed transconjugants were assumed to be derepressed. This transitory derepression of conjugative transfer gene expression in transconjugants was suggested to last for ~6 generations before fertility inhibition is re-established in the transconjugant cell (Cullum et al., 1978; Lundquist and Levin, 1986; Willetts, 1974). A similar interpretation was obtained when dynamics of plasmid transfer was monitored in agar surface cultures using R1 and the mutated derepressed R1*drd*19 derivative (Simonsen, 1990). Transconjugant yield was found to be not significantly different for R1 and R1*drd*19 under conditions of very low initial cell density. Simonsen hypothesized that under these conditions most transconjugants receive the plasmid from another transconjugant cell. Transitory derepression of sex-pilus synthesis in R1 transconjugants would allow plasmid spread throughout a recipient colony at a rate similar to that of R1*drd*19. In summary, repressed plasmids appear to have evolved a strategy to increase plasmid invasion of a bacterial population once the conditions are favorable. In light of the relevance of repressed plasmids for resistance spread in *Enterobacteriaceae*, it is surprising that this hypothesis was never confirmed by more direct experimental data.

Over the last decade, advances in reporter gene technology have provided new insights into the extent and spatial frequencies of horizontal gene transfer in vitro and in natural environments (Sorensen et al., 2005). Application of this methodology involves integration of genes encoding reporter proteins such as green fluorescent protein in the conjugative plasmid to be studied. In this way, fate of plasmids in a bacterial community can be monitored in situ non-destructively. Using this approach, spread of different IncP-1 and IncP-9 plasmids was monitored in a variety of environments including agar-surface grown colonies (Christensen et al., 1996; Fox et al., 2008; Krone et al., 2007), biofilm model systems (Christensen et al., 1998; Haagensen et al., 2002; Hausner and Wuertz, 1999; Krol et al., 2011; Ong et al., 2009; Seoane et al., 2011), freshwater microcosms (Dahlberg et al., 1998) or plant leaves (Normander et al., 1998). Interestingly, spatial analysis of green fluorescence revealed that invasive spread of IncP plasmids was neither detectable in recipient colonies on agar surfaces nor in recipient microcolonies in flow-chamber biofilms suggesting that local factors limit plasmid transfer (Christensen et al., 1998, 1996; Fox et al., 2008).

We are interested in understanding the extent and relevance of transitory derepression in spread of repressed conjugative plasmids. This study aimed to reveal the local distribution of IncF plasmid transfer in surface-grown colonies. Using a triple-labeling methodology, our in situ data reveals that invasion of recipient colonies by plasmids is limited to a narrow superficial zone independent of the repression type.

2. Materials and methods

2.1. Strains, plasmids, oligos, and media

Escherichia coli strains and plasmids used in this study are listed in Table 1, oligonucleotides are listed in *Table* S1 (Supplementary material). Bacteria were grown in Luria–Bertani (LB) medium or agar containing 5 g NaCl per liter (Bertani, 1951) at 37 °C. Selective media contained antibiotics in the following concentrations: kanamycin (Km) 50 μg ml^{-1}, chloramphenicol (Cm) 10 μg ml^{-1}, ampicillin (Amp) 100 μg ml^{-1}, tetracycline (Tet) 10 μg ml^{-1}, rifampicin (Rif) 50 μg ml^{-1}, and streptomycin (Sm) 100 μg ml^{-1}.

2.2. Agar surface matings

Quantification of R1 and R1*drd*19 transfer on agar surfaces was performed under similar conditions as described previously (Simonsen, 1990) with slight adaptations. In brief, over night cultures (ONC-s) of *E. coli* CSH26 donors and CSH26Sm recipients were normalized to an OD_{600} of 0.2 in saline. Twenty-five microliters of donor and recipient suspensions of the undiluted samples as well as four serial dilutions (dilution factor 10) were transferred to an LB agar plate and spread over the entire agar surface (63.7 cm^2) using five sterile glass beads (ø4 mm; Assistent, Germany). After incubation for 22 h at 37 °C, the central 2 × 2 cm agar block of each plate was transferred to a 15 ml tube containing 10 ml of saline. Following vortexing for 30 s to recover the cells from the agar surface, dilutions were spread on selective LB agar plates to quantify donor, recipient, and transconjugant cells. To make sure that transconjugants arise from cultivation on the agar plate, similar control experiments were performed as described previously (Simonsen, 1990).

Agar surface matings for in situ monitoring were prepared as described above except that SAR08 donor cells and SAR20Rif recipient cells were inoculated at initial cell densities of 40 and 400 cfu cm^{-2}. Fluorescence microscopy was performed after 18 h of incubation at 37 °C. For cultures subject to confocal laser microscopy, appropriate dilutions of the normalized donor and recipient suspensions were spread on a microscope slide covered with 1 ml of LB agar. To enable triple color labeling, non-transferable low-copy plasmid pAR179 expressing red-fluorescent DsRed2.T3 under control of a ribosomal promoter was present in SAR08 donor cells.

2.3. Chromosmal insertions in CSH26

A $lacI^{Q1}$ expression cassette was generated by PCR amplification from $lacI^{Q}$ of pSM1431 using primers ar007 and ar008, digestion with BamHI and KpnI and ligation to BamHI and KpnI cut pUC18Not to create pAR64. The $lacI^{Q1}$

Table 1
Strains and plasmids used in this study.

Strain/plasmid	Construction/characteristics[a]	Reference/source
Strain		
CSH26	*ara*, Δ(*lac-pro*), *thi*	Regine Kahmann, Berlin
CSH26Sm	*ara*, Δ(*lac-pro*), *thi*, Sm^R	Reisner et al. (2003)
DY330Nal	W3110 Δ*lacU169* λ*cI857* Δ(*cro-bioA*)	Reisner et al. (2002)
JM101	*supE*, *thi*, Δ(*lac-pro AB*), [F′ *traD36*, *proAB*, $lacI^Q$, Δ(*lacZ*)M15]	Regine Kahmann, Berlin
MT102	*araD139*, (*ara-leu*)7697, Δ*lac*, *thi*, *hsdR*, *mcr*, *rpsL*	Andersen et al. (2001)
SAR08	CSH26 $attB_\lambda$::*bla-lacI*^{Q1}, Amp^R	This study
SAR18	CSH26 $attB_\lambda$::*bla*-$P_{A1/04/03}$-*gfpmut3b**-T_0, Amp^R	Reisner et al. (2003)
SAR20	CSH26 $attB_\lambda$::*bla*-$P_{A1/04/03}$-*yfp**-T_0, Amp^R	Reisner et al. (2003)
SAR20Rif	Spontaneous Rif^R variant of SAR20	This study
Plasmid		
R1	IncFII, Amp^R, Cm^R, Km^R, Sm^R	Helmut Schwab, Graz
R1*drd*19	IncFII, Amp^R, Cm^R, Km^R, Sm^R, *drd*	Helmut Schwab, Graz
pOX38Km	IncFI, Km^R, *aph* of Tn*5* in 45.5 kb F *Hin*dIII fragment	Chandler and Galas (1983)
pLG221	IncI1, ColIb-P9*drd*1 *cib*::Tn*5*, Km^R	Chatfield et al. (1982)
pLG272	IncI1, ColIb-P9 *cib*::Tn*5*, Km^R	Chatfield et al. (1982)
pSU2007	IncW, Km^R, Tp^R, *aph* of Tn*5* in *Eco*RI-*Sal*I fragment of R388	Martinez and de la Cruz (1988)
pAR64	$lacI^{Q1}$ in pUC18Not, Amp^R	This study
pAR92	*cat*-$P_{A1/04/03}$-*cfp**-T_0 cassette in pTP809, Amp^R, Cm^R	Reisner et al. (2002)
pAR93	*cat*-$P_{A1/04/03}$-*yfp**-T_0 cassette in pTP809, Amp^R, Cm^R	Reisner et al. (2002)
pAR117	R1 *cat*::*tetRA*-$P_{A1/04/03}$-*cfp**-T_0	Reisner et al. (2002)
pAR119	R1*drd*19 *cat*::*tetRA*-$P_{A1/04/03}$-*cfp**-T_0	Reisner et al. (2002)
pAR120	R1*drd*19 *cat*::*tetRA*-$P_{A1/04/03}$-*yfp**-T_0	Reisner et al. (2002)
pAR110	pLG221 *aph*::*cat*-$P_{A1/04/03}$-*cfp**-T_0	This study
pAR112	pLG272 *aph*::*cat*-$P_{A1/04/03}$-*cfp**-T_0	This study
pAR108	pOX38Km *aph*::*cat*-$P_{A1/04/03}$-*cfp**-T_0	Reisner et al. (2003)
pAR145	pSU2007 *aph*::*cat*-$P_{A1/04/03}$-*cfp**-T_0	This study
pAR179	$P_{rrnB\text{-}P1}$-dsred2.T3*-T_0 cassette, p15a replicon, Cm^R	Sherlock et al. (2004)
pSM1431	pUC18 carrying $lacI^Q$, Amp^R	Christensen et al. (1998)
pUC18Not	Cloning vector, Amp^R	de Lorenzo and Timmis (1994)

[a] The symbol :: indicates the insertion of the relevant cassette.

carrying NotI fragment of pAR64 was ligated to the *bla-attP* containing NotI-digested pLDR11. Integration-proficient *E. coli* CSH26 [pLDR8] cells were transformed with each ligation mixture as previously described (Reisner et al., 2002). The proper insertion at the $attB_\lambda$ locus of the resulting strains SAR08 was verified by PCR. Genetic manipulation of strain did not affect conjugation frequencies compared to wildtype (data not shown).

2.4. Recombinogenic engineering of conjugative plasmids

Construction of pAR110, pAR112, and pAR145 was performed as previously described (Reisner et al., 2002). Targeting DNA was amplified in a two-step process from plasmids pAR92 using primers ar011 and ar012 to introduce 41 bp ends homologous to the corresponding target sequences. The 3′-ends of the homology extensions correspond to the following base positions on Tn*5* (Accession number U00004): ar011, 1644; ar012, 2205C. The proper insertion of the cassettes was verified by PCR by combining primers homologous to the regions flanking the target sites *aph* (ar009 and ar010) with primers that bind within the insertions (Ucas(C) and Dcas(C)).

2.5. Microscopy

Epifluorescence microscopy was performed using a Zeiss Axioskop epifluorescence microscope equipped with a mercury vapor lamp (HBO50, OSRAM) and Chroma filter sets DsRed, EGFP, ECFP, and EYFP (Chroma techn. Corp., USA). Pictures were acquired using a VISICAM camera (Visitron Systems, GmbH., Germany) and the MetaMorph® Imaging Package 4.0. Confocal laser microscopy was performed using a LEICA AOBS SP2 MP microscope using appropriate laser settings for simultaneous monitoring of Cfp∗, Yfp∗ and Dsred2.T3 fluorophores. Z-stacks of overlay images representing all three detection channels were processed to video sequences.

2.6. Swarming assay

To monitor the effect of R1 or R1*drd*19 carriage on swarming motility of the *E. coli* CSH26 derivative SAR18, 1 μl of ONC-s normalized to an OD_{600} of 0.5 were spotted on agar plates prepared with AB minimal medium (Clark and Maaløe, 1967) supplemented with glucose (0.4%), proline (10 μg ml^{-1}), and thiamine (1 μg ml^{-1}) solidified with agar at a final concentration of 0.7 % (w/v). Expansion of colonies was recorded after 65 h of incubation at 30 °C in a closed box containing a water reservoir to avoid extensive drying of plates.

3. Results

3.1. At low initial cell densities R1 and R1drd19 spread with similar frequencies among isogenic E. coli CSH26 strains on agar surfaces

To confirm the suitability of the laboratory *E. coli* strain CSH26 for studying in situ transfer of R1 and R1*drd*19

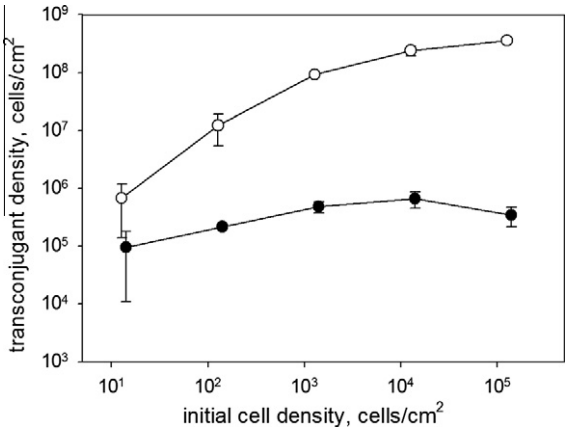

Fig. 1. Transconjugant densities of surface matings with plasmids R1 and R1*drd*19 depend on initial cell density. Transconjugant densities (cfu cm^{-2} of agar; $M \pm SE$, $n = 4$) are shown for R1 (filled circles) and R1*drd*19 (open circles) after 22 h of growth on LB agar plates using different initial cell densities.

under conditions that favor transitory derepression of conjugative transfer gene expression, we performed surface matings with these plasmids under similar conditions as described previously using varying initial cell densities (Simonsen, 1990). The dependence of transconjugant yield of R1 and R1*drd*19 on initial cell density highly resembled the data reported by Simonsen (Fig. 1). Reduction of initial cell density by four orders of magnitude reduced R1*drd*19 transconjugant yield 530-fold whereas R1 transconjugant yield was only reduced by a factor of 3.5. At lowest initial density of 13 cells cm^{-2}, transconjugant yields of R1 and R1*drd*19 were not significantly different ($P = 0076$). The number of total cells recovered from surface matings was constant independent of initial cell density (data not shown).

Resemblance of our data to Lone Simonsen's data enabled us to test the proposed hypothesis that at low initial cell densities transitory derepression of sex-pilus synthesis in R1 transconjugants would allow plasmid spread throughout a recipient colony at a rate similar to that of R1*drd*19 (Simonsen, 1990). To monitor transitory derepression in situ, we chose therefore to use low initial cell densities.

3.2. A dual-color-labeling strategy to monitor plasmid spread between E. coli in situ

A zygotic induction strategy was applied to monitor R1 and R1*drd*19 spread through recipient colonies in situ as described previously for monitoring plasmid transfer between *Pseudomonas* spp. (Christensen et al., 1998). In our system, donor cells expressing a chromosomally encoded LacI repressor and carrying $P_{A1/04/03}$-*cfp*∗ tagged conjugative plasmids are combined with recipient cells lacking a functional LacI protein. Since cfp∗ expression from $P_{A1/04/03}$ is under tight control of the LacI repressor in the donor cells, cyan fluorescence can only emerge after transfer of the tagged conjugative plasmid to a recipient cell. When we tested this approach in *E. coli* K-12 strains, we found that even in the presence of *lacI* or a $lacI^Q$ allele in the *E. coli* chromosome, partial fluorescent reporter expression from a tagged R1*drd*19 derivative was detectable at colony and single cell levels by epifluorescence microscopy (Supplementary material Fig. S1). We therefore inserted a $lacI^{Q1}$ variant in the $attB_\lambda$ site of *E. coli* CSH26 that is reported to result in stronger LacI expression (Glascock and Weickert, 1998). Indeed, no residual fluorescence was detectable when a fluorescently tagged R1*drd*19 derivative was maintained in the resulting donor strain SAR08 (Supplementary material Fig. S1). Integration of $lacI^{Q1}$ did not affect the capability to act as plasmid donor compared to wildtype CSH26 (data not shown).

To differentiate donor from recipient cells, a $P_{A1/04/03}$-*yfp*∗-tagged *E. coli* CSH26 strain designated as SAR20 was utilized as recipient strain. Transconjugant cells are therefore indicated by expressing both cyan and yellow fluorescence.

3.3. In situ monitoring of R1 and R1drd19 spread through recipient colonies reveals distinct plasmid transfer phenotypes

To monitor the R1 and R1*drd*19 spread in situ, SAR08 donor and SAR20Rif recipient strains were co-inoculated on dried agar surfaces at average densities of 40 and 400 cfu cm^{-2}. After 18 h of incubation, epifluorescence microscopy was used to detect plasmid fate in colliding colonies (Fig. 2). Independent of the inoculation density, two different plasmid dissemination patterns were observed. Transfer of the R1 derivative pAR117 was manifested by small foci of transconjugants randomly localized within regions where individual donor and recipient colonies collided through expansion. For the derepressed R1*drd*19 derivative pAR119, distinct continuous zones of transconjugants were visible wherever a non-fluorescent donor colony was directly adjacent to a yellow fluorescent recipient colony. Prolonged cultivation for 64 h did not result in a detection of transconjugants further within the recipient colony (data not shown) that would have indicated a stronger invasion of the plasmids throughout a recipient colony.

These observations suggested that the number of microscopically observed transconjugants under the tested conditions is inversely proportional to the repression status of conjugative pilus synthesis in the original donors, however, the extent of plasmid invasion into recipient colonies appeared to be similar for both R1 and R1*drd*19.

3.4. Confocal laser microscopy reveals limited plasmid invasion into recipient colonies

To reveal the extent of plasmid invasion into recipient colonies with greater precision, agar surface matings were subject to confocal laser scanning microscopy (CLSM). To visualize donor cells and to guarantee that the experimental conditions support fluorescent protein synthesis and maturation in the agar-grown cells, SAR08 donor cells expressing a red-fluorescent protein were used. CLSM analysis of apposed donor and recipient colonies revealed a detailed view of the spatial occurrence of transconjugant cells within the top 40–70 μm of the surface population (Fig. 3, Supplementary material Video S1 and

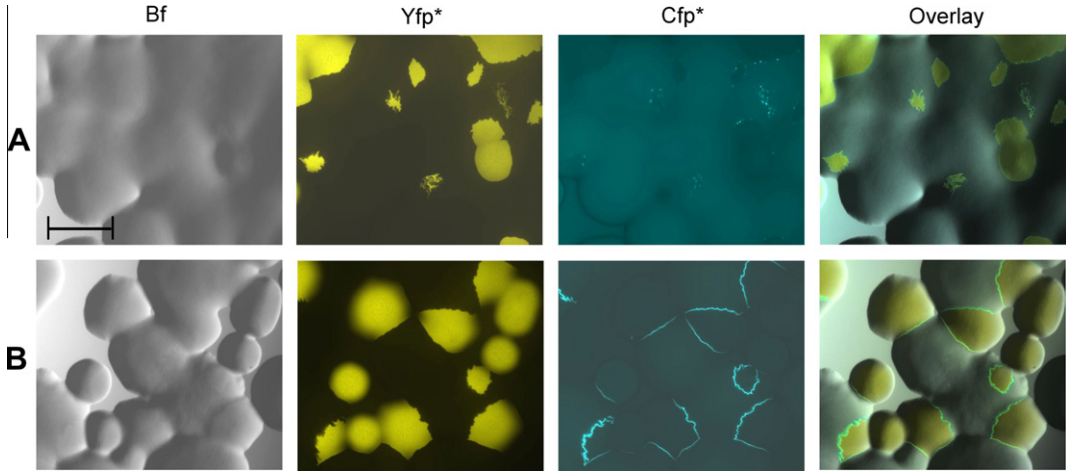

Fig. 2. In situ monitoring of R1 and R1*drd*19 DNA transfer on agar surfaces. Columns represent bright field (BF), epifluorescence (Yfp* and Cfp*), and overlay images of representative areas of agar surfaces inoculated with 400 cfu cm^{-2} of *E. coli* SAR20Rif recipient and SAR08 donor cells carrying the R1 derivative pAR117 (A) or the R1*drd*19 derivative pAR119 (B) after 18 h of incubation at 37 °C. The scale bar represents 400 μm.

S2). In agreement with the epifluorescence analysis, R1 transconjugants formed localized foci of cells that extended through the visualized section of the colonies. R1*drd*19 transconjugants formed a continuous layer between donor and recipient cells. We concluded that neither R1 nor R1*drd*19 derivatives were able to spread beyond the first five recipient cell layers adjacent to the donor cells.

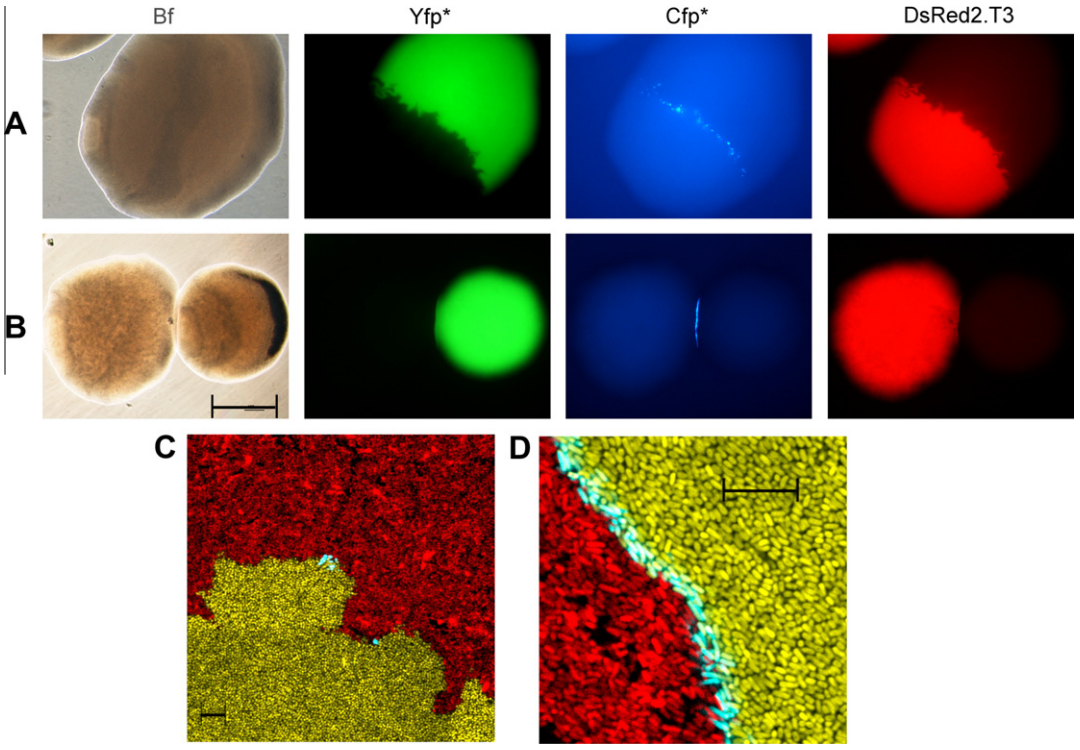

Fig. 3. CLSM reveals the single cell level spatial organization of R1 and R1*drd*19 transconjugants emerging during mating on agar surfaces. Columns represent bright field (BF) and epifluorescence (Yfp*, Cfp*, and Dsred2.T3) images of representative areas of agar surfaces inoculated with 400 cfu cm^{-2} of *E. coli* SAR20 recipient and SAR08[pAR179] donor cells carrying the R1 derivative pAR117 (A) or the R1*drd*19 derivative pAR119 (B) after 18 h of incubation at 37 °C. CLSM analysis of representative mating areas is displayed as overlay images for pAR117 (C) and pAR119 (D). Yfp fluorescence is displayed in green color. The scale bars represent 400 μm (A–B) and 10 μm (C–D), respectively.

3.5. Transfer of other prototypical IncF, IncI, and IncW plasmids is also limited to the contact zone

To investigate whether other derepressed and repressed conjugative plasmids would reveal a similar pattern of plasmid spread throughout recipient colonies we extended our in situ analysis to other prototypical conjugative plasmids that were tagged with a $P_{A1/04/03}$-*cfp** expression cassette: plasmid F (IncF, derepressed), ColIb-P9 (IncI, repressed wildtype plasmid and derepressed mutant) and R388 (IncW, derepressed). Epifluorescence analysis of mating zones of colliding SAR08 donor colonies and SAR20Rif recipient colonies revealed that distribution of transconjugant cells resembled the patterns observed for R1 and R1*drd*19 depending on the repression state of the tested prototype plasmid (Supplementary material Fig. S2). Transconjugants of the derepressed ColIb-P9 derivative were detected as small foci localized randomly within regions where individual donor and recipient colonies were spatially apposed. Derepressed IncF, IncI, and IncW derivatives produced continuous zones of transconjugants along the line of intersection. We concluded that plasmid spread is generally limited to a narrow zone of recipient cells.

3.6. Derepressed R1drd19 limits swarming motility in E. coli CSH26

Since in situ observations using R1 and R1*drd*19 did not support a pronounced spread of plasmids into the recipient colony, we evaluated whether other effects could account for the similar yield of transconjugant observed at low initial densities during surface matings (1). Since Barrios and co-workers recently described that carriage of R1*drd*19 eliminated swimming motility of *E. coli* laboratory strains (Barrios et al., 2006), we reasoned that reduced colony expansion of R1*drd*19 donors might lead to fewer colony collisions and, thus, a transconjugant yield resembling the more motile R1 donors. Indeed, we found that swarming motility of a *E. coli* CSH26 derivative over agar surfaces containing a lower concentration of agar (0.7%) was strongly affected by the carriage of R1*drd*19 (Fig. 4). Under these experimental conditions motility of host cells lacking a plasmid enabled the colony to expand to a diameter of ~3 cm. Whereas carriage of R1 did not alter expansion of the colony, R1*drd*19 colonies did not exceed a diameter of ~1 cm. However, comparison of swarming motility using LB agar plates containing standard concentrations of agar (1.5%) did not reveal pronounced differences in colony expansion due to plasmid carriage (data not shown).

We concluded that R1*drd*19 carrying cells are affected in motility and surface migration under certain conditions, however, the data do not resolve whether this contributes to reduced transconjugant yield at low initial cell densities.

4. Discussion

Here we present the first in situ analysis of the fate of a repressed and derepressed IncF plasmids in an agar surface model system using laboratory K-12 strains. Based on the observation of similar plasmid transfer at low initial cell densities monitored by colony counts, we expected similar invasion of recipient colonies by repressed and derepressed R1 derivatives due to transitory derepression in the transconjugant cells. High resolution in situ analysis revealed different distribution patterns of transconjugant cells for R1 and R1*drd*19, however, plasmid invasion did not reach beyond the first five recipient cell layers at the donor/recipient interface for both plasmids. Given that the zygotic induction strategy does not allow differentiation of horizontal or vertical plasmid transmission, we are unable to determine the exact level of plasmid donor activity from newly formed transconjugants. The low plasmid invasion into recipient colonies does not support the model that plasmid spread occurs throughout the recipient colony, as originally proposed (Simonsen, 1990).

Extension of in situ analysis to other prototypical plasmids of the IncF, IncI, and IncW families revealed similarly limited levels of recipient colony invasion. These observations are in agreement with previous studies monitoring IncP plasmid invasion in agar colonies and biofilm setups (Christensen et al., 1998, 1996; Fox et al., 2008). It therefore appears that in these model systems plasmid invasion is generally limited independent of the type of conjugative transfer system and sex-pilus characteristics. In a study to reveal factors that limit IncP plasmid invasion in a similar experimental setup, Fox and co-workers found that replenishment of nutrients increased plasmid invasion and regular disturbance of the spatial organization strongly improved plasmid invasiveness (Fox et al., 2008). Thus, even derepressed expression of transfer genes cannot surpass the limitations of nutrient starvation and a low level of mixing due to the dense packing of cells in agar-grown colonies.

In situ analysis of repressed IncF and IncI plasmid transfer also revealed that only few cells at the donor/recipient

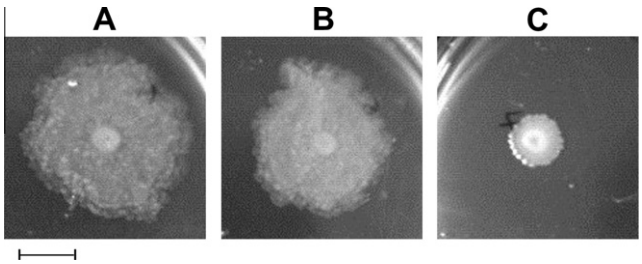

Fig. 4. Surface motility of CSH26 is affected by R1*drd*19. Agar surface motility of the CSH26 derivative SAR18 (A), SAR18 [R1] (B) and SAR18 [R1*drd*19] (C) after 65 h of incubation are shown. The scale bar represents 1 cm.

interface are able to escape repression of transfer gene expression allowing transfer of the plasmids to neighboring recipient cells. Simulation of repressed plasmid spread using mathematical modeling approaches to fit the experimental data might help to unravel the local requirements for this escape. A previous attempt to fit the agar surface mating results from Simonsen (1990) did not reveal realistic parameters for R1 dissemination (Krone et al., 2007), nevertheless modeling attempts for derepressed plasmids fitted well with the observation that R1*drd*19 spread is dependant on initial cell densities (Krone et al., 2007; Zhong et al., 2012). New modeling attempts using an experimental system that enables better nutrient availability and/or spatial mixing might also allow simulation of the transitory derepression phenomenon. Our results also suggest that variation of cell motility based on the transfer expression state would be a relevant parameter to include in these simulations. Together, these efforts will help to uncover the mechanisms that enable the successful spread of repressed plasmids in clinically relevant bacteria.

Acknowledgments

We thank B. Glick, S. Molin, B. Wanner and B. Wilkins for providing plasmids. This work was supported by the Austrian Science Fund (FWF): P16722 and P18607 (to E.Z.); P21434 and J2250 (to A.R.).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.plasmid.2012.01.001.

References

Andersen, J.B. et al., 2001. *Gfp*-Based *N*-Acyl homoserine-lactone sensor systems for detection of bacterial communication. Appl. Environ. Microbiol. 67, 575–585.

Barrios, A.F. et al., 2006. Hha, YbaJ, and OmpA regulate *Escherichia coli* K12 biofilm formation and conjugation plasmids abolish motility. Biotechnol. Bioeng. 93, 188–200.

Bergstrom, C.T. et al., 2000. Natural selection, infectious transfer and the existence conditions for bacterial plasmids. Genetics 155, 1505–1519.

Bertani, G., 1951. Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*. J. Bacteriol. 62, 293–300.

Bradley, D.E. et al., 1980. Specification of surface mating systems among conjugative drug resistance plasmids in *Escherichia coli* K-12. J. Bacteriol. 143, 1466–1470.

Canton, R. et al., 2008. Prevalence and spread of extended-spectrum beta-lactamase-producing *Enterobacteriaceae* in Europe. Clin. Microbiol. Infect. 14 (Suppl. 1), 144–153.

Chandler, M., Galas, D.J., 1983. Cointegrate formation mediated by Tn*9*. II. Activity of IS*1* is modulated by external DNA sequences. J. Mol. Biol. 170, 61–91.

Chatfield, L.K. et al., 1982. DNA primase of plasmid CollB is involved in conjugal DnA synthesis in donor and recipient bacteria. J. Bacteriol. 152, 1188–1195.

Christensen, B.B. et al., 1998. Establishment of new genetic traits in a microbial biofilm community. Appl. Environ. Microbiol. 64, 2247–2255.

Christensen, B.B. et al., 1996. Bacterial plasmid conjugation on semi-solid surfaces monitored with the green fluorescent protein (GFP) from *Aequorea victoria* as a marker. Gene 173, 59–65.

Clark, D.J., Maaløe, Z., 1967. DNA replication and the division cycle in *Escherichia coli*. J. Mol. Biol. 23, 99–112.

Coque, T.M. et al., 2008. Dissemination of clonally related *Escherichia coli* strains expressing extended-spectrum beta-lactamase CTX-M-15. Emerg. Infect. Dis. 14, 195–200.

Cullum, J. et al., 1978. The spread of plasmids in model populations of *Escherichia coli* K12. Plasmid. 1, 545–556.

Dahlberg, C. et al., 1998. *In situ* detection of high levels of horizontal plasmid transfer in marine bacterial communities. Appl. Environ. Microbiol. 64, 2670–2675.

de Lorenzo, V., Timmis, K.N., 1994. Analysis and construction of stable phenotypes in gram-negative bacteria with Tn*5*- and Tn*10*-derived minitransposons. Methods Enzymol. 235, 386–405.

Fox, R.E. et al., 2008. Spatial structure and nutrients promote invasion of IncP-1 plasmids in bacterial populations. ISME J. 2, 1024–1039.

Frost, L.S., Koraimann, G., 2010. Regulation of bacterial conjugation: balancing opportunity with adversity. Future Microbiol. 5, 1057–1071.

Glascock, C.B., Weickert, M.J., 1998. Using *lacI*Q1 to control expression of genes on high-copy-number plasmids in *Escherichia coli*. Gene. 223, 221–231.

Haagensen, J.A.J. et al., 2002. In situ detection of horizontal transfer of mobile genetic elements. FEMS Microbiol. Ecol. 42, 261–268.

Hausner, M., Wuertz, S., 1999. High rates of conjugation in bacterial biofilms as determined by quantitative in situ analysis. Appl. Environ. Microbiol. 65, 3710–3713.

Hawkey, P.M., 2008. Molecular epidemiology of clinically significant antibiotic resistance genes. Br. J. Pharmacol. 153 (Suppl. 1), S406–S413.

Koraimann, G. et al., 1991. Repression and derepression of conjugation of plasmid R1 by wild-type and mutated *finP* antisense RNA. Mol. Microbiol. 5, 77–87.

Koraimann, G. et al., 1996. The FinOP repressor system of plasmid R1: analysis of the antisense RNA control of *traJ* expression and conjugative DNA transfer. Mol. Microbiol. 21, 811–821.

Krol, J.E. et al., 2011. Increased transfer of a multidrug resistance plasmid in *Escherichia coli* biofilms at the air–liquid interface. Appl. Environ. Microbiol. 77, 5079–5088.

Krone, S.M. et al., 2007. Modelling the spatial dynamics of plasmid transfer and persistence. Microbiology 153, 2803–2816.

Llosa, M. et al., 2009. Bacterial type IV secretion systems in human disease. Mol. Microbiol. 73, 141–151.

Lundquist, P.D., Levin, B.R., 1986. Transitory derepression and the maintenance of conjugative plasmids. Genetics 113, 483–497.

Martinez, E., de la Cruz, F., 1988. Transposon Tn*21* encodes a *RecA*-independent site-specific integration system. Mol. Gen. Genet. 211, 320–325.

Normander, B. et al., 1998. Effect of bacterial distribution and activity on conjugal gene transfer on the Phylloplane of the bush bean (*Phaseolus vulgaris*). Appl. Environ. Microbiol. 64, 1902–1909.

Novais, A. et al., 2007. Emergence and dissemination of *Enterobacteriaceae* isolates producing CTX-M-1-like enzymes in Spain are associated with IncFII (CTX-M-15) and broad-host-range (CTX-M-1, -3, and -32) plasmids. Antimicrob. Agents Chemother. 51, 796–799.

Ong, C.L. et al., 2009. Conjugative plasmid transfer and adhesion dynamics in an *Escherichia coli* biofilm. Appl. Environ. Microbiol. 75, 6783–6791.

Polzleitner, E. et al., 1997. TraM of plasmid R1 controls transfer gene expression as an integrated control element in a complex regulatory network. Mol. Microbiol. 25, 495–507.

Queenan, A.M., Bush, K., 2007. Carbapenemases: the versatile beta-lactamases. Clin Microbiol Rev. 20, 440–458.

Reisner, A. et al., 2003. Development and maturation of *Escherichia coli* K-12 biofilms. Mol. Microbiol. 48, 933–946.

Reisner, A. et al., 2002. Recombinogenic engineering of conjugative plasmids with fluorescent marker cassettes. FEMS Microbiol. Ecol. 42, 251–259.

Seoane, J. et al., 2011. An individual-based approach to explain plasmid invasion in bacterial populations. FEMS Microbiol Ecol. 75, 17–27.

Sherlock, O. et al., 2004. Novel roles for the AIDA adhesin from diarrheagenic *Escherichia coli*: cell aggregation and biofilm formation. J. Bacteriol. 186, 8058–8065.

Simonsen, L., 1990. Dynamics of plasmid transfer on surfaces. J. Gen. Microbiol. 136, 1001–1007.

Sorensen, S.J. et al., 2005. Studying plasmid horizontal transfer in situ: a critical review. Nat. Rev. Microbiol. 3, 700–710.

Su, L.H. et al., 2008. An epidemic of plasmids? Dissemination of extended-spectrum cephalosporinases among *Salmonella* and other *Enterobacteriaceae*. FEMS Immunol. Med. Microbiol. 52, 155–168.

Walther-Rasmussen, J., Hoiby, N., 2002. Plasmid-borne AmpC beta-lactamases. Can. J. Microbiol. 48, 479–493.

Willetts, N.S., 1974. The kinetics of inhibition of F*lac* transfer by R100 in *E. coli*. Mol. Gen. Genet. 129, 123–130.

Zhong, X. et al., 2012. On the meaning and estimation of plasmid transfer rates for surface-associated and well-mixed bacterial populations. J. Theor. Biol. 294C, 144–152.